

## Cellular/Molecular

# Wnt-7a Induces Presynaptic Colocalization of $\alpha 7$ -Nicotinic Acetylcholine Receptors and Adenomatous Polyposis Coli in Hippocampal Neurons

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Nicotinic acetylcholine receptors (nAChRs) contribute significantly to hippocampal function.  $\alpha 7$ -nAChRs are present in presynaptic sites in hippocampal neurons and may influence transmitter release, but the factors that determine their presynaptic localization are unknown. We report here that *Wnt-7a*, a ligand active in the canonical *Wnt* signaling pathway, induces dissociation of the adenomatous polyposis coli (APC) protein from the  $\beta$ -catenin cytoplasmic complex and the interaction of APC with  $\alpha 7$ -nAChRs in hippocampal neurons. Interestingly, *Wnt-7a* induces the relocation of APC to membranes, clustering of APC in neurites, and coclustering of APC with different, presynaptic protein markers. *Wnt-7a* also increases the number and size of coclusters of  $\alpha 7$ -nAChRs and APC in presynaptic terminals. These short-term changes in  $\alpha 7$ -nAChRs occur in the few minutes after ligand exposure and involve translocation to the plasma membrane without affecting total receptor levels. Longer-term exposure to *Wnt-7a* increases nAChR  $\alpha 7$  subunit levels in an APC-independent manner and increases clusters of  $\alpha 7$ -nAChRs in neurites via an APC-dependent process. Together, these results demonstrate that stimulation through the canonical *Wnt* pathway regulates the presynaptic localization of APC and  $\alpha 7$ -nAChRs with APC serving as an intermediary in the  $\alpha 7$ -nAChR relocation process. Modulation by *Wnt* signaling may be essential for  $\alpha 7$ -nAChR expression and function in synapses.

**Key words:** nAChR; APC; synapse; *Wnt*; *Wnt* target gene; neurons

## Introduction

In the CNS,  $\alpha 7$ -nicotinic acetylcholine receptors ( $\alpha 7$ -nAChRs) are involved in several aspects of brain function.  $\alpha 7$ -nAChRs affect neuronal development (Role and Berg, 1996), learning, and memory (Levin and Simon, 1998). Because of their high permeability to calcium ions,  $\alpha 7$ -nAChRs influence synaptic efficacy and induction of long-term potentiation (Vernino et al., 1992).  $\alpha 7$ -nAChRs have been implicated in a wide variety of neuronal diseases, including schizophrenia (Freedman et al., 2003), bipolar disorder, Parkinson's disease, and Alzheimer's disease (AD) (Banerjee et al., 2000; Kem, 2000).

In hippocampal neurons,  $\alpha 7$ -nAChRs can be found at presynaptic terminals, at which they colocalize with synaptotagmin, thus explaining their role in neurotransmitter release at both GABAergic and glutamatergic nerve terminals (Gray et al., 1996; Radcliffe and Dani, 1998; Alkon and Albuquerque, 2001; Kawai et al., 2002; Zago et al., 2006). The factors that control  $\alpha 7$ -nAChR distribution and influence its presynaptic localization are still unknown.

Canonical *Wnt* signaling is essential for neuronal development and the maintenance of the developing nervous system (Patapoutian and Reichardt, 2000), and it has recently been implicated in adult hippocampal neurogenesis (Lie et al., 2005). During the last several years, work in our laboratory has demonstrated that the activation of *Wnt* signaling prevents amyloid- $\beta$  (A $\beta$ ) neurotoxicity in hippocampal neurons (Garrido et al., 2002; De Ferrari et al., 2003; Alvarez et al., 2004; Quintanilla et al., 2005).

Activation of *Wnt* signaling by a specific, canonical *Wnt* ligand involves dissociation of  $\beta$ -catenin from a complex also containing axin, casein kinase, glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) and the adenomatous polyposis coli (APC) protein. In the absence of *Wnt* activation,  $\beta$ -catenin in the complex is phosphorylated, ubiquitinated, and degraded in the proteasome (Aberle et al., 1997). However, *Wnt* ligand action promotes  $\beta$ -catenin dis-

Received May 2, 2006; revised March 19, 2007; accepted March 24, 2007.

This work was supported by grants from Fondo de Investigación Avanzada en Áreas Prioritarias (13980001) and the Millennium Institute for Fundamental and Applied Biology (N.C.I.); by predoctoral fellowships from Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) (G.G.F., M.C.) and from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (A.S.V.); by grants from CONICET, Fondo Nacional para las Ciencias y la Tecnología, and Universidad Nacional del Sur (F.J.B.); by a grant from Secretaría de Ciencia y Tecnología-CONICYT (N.C.I., F.J.B.); by National Institutes of Health Grants DA015389 and NS040417; by Arizona Biomedical Research Commission Grant 9615; and by the Barrow Neurological Foundation (R.J.L.). We thank Drs. Patricia Salinas, Jeremy Nathans, and Randall Moon for their kind gifts of different constructs.

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DOI:10.1523/JNEUROSCI.3934-06.2007

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sociation from the destruction complex, thus enhancing  $\beta$ -catenin stabilization, and translocation of  $\beta$ -catenin to the nucleus, in which it interacts with Tcf/LEF transcription factors to activate the expression of *Wnt* target genes (Nusse and Varmus, 1992). The latter include *c-myc*, *engrailed-2*, *cyclooxygenase-2*, *neurogenin-1*, and other genes (Moon et al., 2004).

APC, a component of the  $\beta$ -catenin degradation complex, has been suggested to play a role in synapses. In particular, skeletal muscle APC is required for agrin-induced AChR clustering at the neuromuscular junction (Wang et al., 2003), and APC maintains the surface levels as well as the clustering of postsynaptic  $\alpha 3^*$ -nAChRs in chick ciliary ganglion neurons (Temburni et al., 2004). In hippocampal neurons, APC is expressed at high levels in the cytoplasm, in which it interacts with  $\beta$ -catenin (Brakeman et al., 1999). However, APC also has been found at presynaptic sites, at which it colocalizes with synaptotagmin, a synaptic vesicle protein (Matsumine et al., 1996).

Function(s) of APC in presynaptic sites and mechanisms by which  $\alpha 7$ -nAChRs localize to presynaptic sites are unknown. Here, we investigated roles of *Wnt* canonical signaling in the localization of APC in presynaptic sites and in the interaction of APC with  $\alpha 7$ -nAChRs.

## Materials and Methods

**Constructs.** Control and mouse APC small interfering RNA (siRNA) were from Santa Cruz Biotechnology (Santa Cruz, CA), green fluorescent protein (GFP) was from Clontech (Mountain View, CA); HA-*Wnt-7a* was a gift from Dr. P. Salinas (University College London, London, UK), and HA-sFRP was a gift from Dr. Jeremy Nathans (Johns Hopkins University School of Medicine, Baltimore, MD).

**Hippocampal neuronal cultures and transfection.** Hippocampal neurons were obtained from Sprague Dawley rats at embryonic day 18. Hippocampi were dissected, and primary cultures were prepared as described previously (Cáceres et al., 1984; Fariás et al., 2004) and maintained in DMEM supplemented with 10% horse serum for 2 h. The culture medium was then substituted with Neurobasal media supplemented with B27, 100  $\mu$ g/ml streptomycin, and 100 U/ml penicillin. Cells were treated for 24 h with 2  $\mu$ M 1- $\beta$ -D-arabinofuranosylcytosine (AraC) on day 3 to reduce the number of proliferating non-neuronal cells. Experiments were performed on day 14 in the presence or absence of different *Wnt-7a* or other entities. For transfection using GFP or siRNA constructs, cultured hippocampal neurons from C57BL mice at embryonic day 18 were prepared as for rat hippocampal cultures. Transfection was performed using the calcium phosphate method for neurons as described previously (Kohrmann et al., 1999) with some modifications. Hippocampal neurons were transfected on day 10 *in vitro*, and experiments were performed beginning on day 12 *in vitro* and involving up to 24 h of exposure to medium lacking or containing *Wnt-7a*.

**Cell line culture.** Human embryonic kidney 293 (HEK-293) cells were maintained in DMEM supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), 100  $\mu$ g/ml streptomycin, and 100 U/ml penicillin. In initial studies, wild-type or transfected SH-EP1 cells expressing human  $\alpha 7$ -nAChRs were studied after being maintained according to previous descriptions (Zhao et al., 2003).

**Conditioned medium containing *Wnt* ligands.** To generate secreted *Wnt* ligands, HEK-293 cells were transiently transfected by calcium phosphate precipitation (Conroy and Berg, 1998) with constant and equal amounts of empty vector pcDNA or pcDNA containing sequences encoding *Wnt-7a* ligand. Transiently transfected HEK-293 cells also were used to produce soluble Frizzled receptor protein (sFRP) coupled to the sequence encoding a hemagglutinin tag. Transiently transfected HEK-293 cells were grown to 85% confluence and maintained in Neurobasal medium supplemented with 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin for 60 h. *Wnt*-conditioned or control media or media containing sFRP (Ahmad-Annuar et al., 2006) were prepared as described previously (Hall et al., 2000; Rosso et al., 2004; Quintanilla et al., 2005; Seto

and Bellen, 2006). *Wnt* secretion was verified by Western blot using an anti-HA antibody (Millipore, Billerica, MA).

**Cell fractionation and Western blot for detection of  $\beta$ -catenin and APC.** Total protein was prepared from primary rat hippocampal neurons lysed in a buffer consisting of (in mM) 10 HEPES, pH 7.9, 1.5 MgCl<sub>2</sub>, 10 KCl, and 1 DTT, supplemented with a protease inhibitor mixture (to achieve final concentrations of 1 mM PMSF, 2  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml pepstatin). Lysates were laid on ice for 30 min and centrifuged at 700  $\times$  g for 5 min at 4°C. Supernatants were centrifuged at 100,000  $\times$  g for 1 h at 4°C, and the pellets (equivalent to membrane or particulate fractions) were resuspended in a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 0.15 mM NaCl, and the above protease inhibitor mixture. The supernatant (equivalent to the cytoplasmic fraction) was precipitated with methanol-chloroform. Equal amounts of protein were resolved using SDS-PAGE (6% polyacrylamide), proteins were transferred to PVDF membranes, and immunoblotting was done using anti- $\beta$ -catenin monoclonal or polyclonal anti-APC antibodies (Santa Cruz Biotechnology). Immune complexes were visualized by reaction with secondary antibodies linked to horseradish peroxidase followed by incubation with enhanced chemiluminescence reagents (Santa Cruz Biotechnology).

**Immunoprecipitation assay.** Treated neurons or control substances were washed twice in ice-cold PBS and lysed in a buffer "A" (25 mM HEPES, pH 7.4, 125 mM NaCl, 25 mM NaF, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1 mM NaVO<sub>3</sub>, and the protease inhibitor mixture). Equal amounts of protein were precleared using protein A-Sepharose for 1 h at 4°C and then incubated with 3  $\mu$ g of antibody against the nAChR  $\alpha 7$  subunit or APC. The immune complexes were affinity precipitated with protein A-Sepharose beads and washed six times with 25 mM HEPES buffer, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM NaF, 1% NP-40, and 1 mM NaVO<sub>3</sub>. The immune complexes were then submitted to SDS-PAGE and analyzed by Western blots with mouse anti-APC, mouse anti- $\beta$ -catenin, mouse anti-transferrin receptor (TF-R), mouse anti-tyrosine receptor kinase B (TrkB), goat anti-postsynaptic density protein-95 (PSD-95), or goat anti-vesicle-associated membrane protein 2 (VAMP-2; Santa Cruz Biotechnology).

**$\alpha 7$ -nAChR pull down.** Total numbers of  $\alpha 7$ -nAChR from hippocampal neurons were obtained by washing cells twice in ice-cold PBS followed by lysis in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with the protease inhibitor mixture described above. Lysates were incubated with 1  $\mu$ g/ml biotinylated  $\alpha$ -bungarotoxin ( $\alpha$ -Btx) for 12 h at 4°C before addition of 40  $\mu$ l of streptavidin-Sepharose beads and incubation for 2 h at 4°C. The complex was washed six times in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 1% Triton X-100, pH 8.  $\alpha 7$ -nAChR-containing samples were resolved by SDS-PAGE (6% polyacrylamide) and subjected to immunoblotting as outlined above but for detection of APC and p-synapsin using specific antibodies. As a control to establish specificity of the  $\alpha 7$ -nAChR pull down, we coincubated the cellular extract and biotinylated  $\alpha$ -Btx mixture in the presence of an excess of nonbiotinylated  $\alpha$ -Btx (10  $\mu$ M) or nicotine (1 mM).

**Pulse chase.** Changes in  $\alpha 7$ -AChR levels at the cell surface were evaluated in hippocampal neurons exposed to *Wnt-7a* for different periods; biotinylated- $\alpha$ -Btx (1  $\mu$ g/ml) was added to cultures of live neurons for 45 min to visualize cell surface  $\alpha 7$ -nAChRs and at 4°C to inhibit nAChR endocytosis (Borroni et al., 2007). Neurons were subsequently lysed in RIPA buffer, and lysates were incubated with streptavidin-Sepharose and washed as described above except that first supernatants were saved to analyze intracellular  $\alpha 7$ -nAChRs. Surface  $\alpha 7$ -nAChRs and first supernatants were resolved using SDS-PAGE (8% polyacrylamide) and analyzed by Western blots with rabbit anti-nAChR  $\alpha 7$  subunit antibody (Santa Cruz Biotechnology).

**Determination of total levels of  $\alpha 7$ -nAChRs and DVL phosphorylation.** Cells were lysed in buffer A. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Forty microgram samples of cell lysates were resolved by SDS-PAGE (8% polyacrylamide) followed by immunoblotting on PVDF membranes using rabbit anti-nAChR  $\alpha 7$  subunit or mouse anti-dishevelled-3 (DVL-3) antibody (Santa Cruz Biotechnology).

**Immunohistochemistry.** Hippocampal neurons were subjected to different treatments while on coverslips inside 24-well plates at a plating density of 30,000 cells/coverslip, fixed with 4% paraformaldehyde in PBS for 45 min, permeabilized with 0.1% Triton X-100, and stained with the following antibodies: rabbit polyclonal anti-nAChR  $\alpha 7$  subunit, rabbit polyclonal anti-APC, goat polyclonal anti-synaptic vesicle protein 2 (SV-2), goat polyclonal anti-p-synapsin, and goat polyclonal anti-synaptotagmin I a/b as presynaptic markers, goat polyclonal anti-PSD-95 as a postsynaptic marker, monoclonal anti-AT8 as an axonal marker, and monoclonal anti-microtubule-associated protein 2 (MAP2a/b) as a dendrite marker followed by Alexa<sup>488</sup>-, Alexa<sup>543</sup>-, or Alexa<sup>633</sup>-conjugated secondary antibodies. Phalloidin coupled to Alexa<sup>633</sup> was used as neurite marker. In some experiments,  $\alpha 7$ -nAChRs were labeled with rhodamine (Rhod)- $\alpha$ -Btx in permeabilized cells. Double-labeling experiments with anti-nAChR  $\alpha 7$  subunit antibody (Santa Cruz Biotechnology) and Rhod- $\alpha$ -Btx- gave coincident labeling patterns, supporting the correlation between the  $\alpha 7$  subunit-like antigens and  $\alpha$ -Btx-binding  $\alpha 7$ -nAChRs. Digital images of neurons on coverslips were captured with a Zeiss (Oberkochen, Germany) confocal microscope using a 63 $\times$ /1.4 numerical aperture oil-immersion objective. To determine the number of clusters within 10 or 100  $\mu$ m of neurite length and the major axis length of each cluster ( $\mu$ m), images were analyzed using the LSM 5 Image Browser. Clusters were defined as puncta of 0.8–3  $\mu$ m in major axis length. For quantification of cluster size, length values were compared with those of control treatments. To identify somatic  $\alpha 7$ -nAChR and determine fluorescence staining intensity and numbers of  $\alpha 7$ -nAChR-containing clusters, 30 soma expressing GFP were examined for each treatment using Image J.

**Colocalization image analysis.** Colocalization analysis was performed on randomly selected images ( $n = 15$ ) using NIH Image J software with the colocalization analysis plug-in. Mander's coefficients ( $M$ ) represent the fraction of pixels in which the two signals overlap or are colocalized (Manders et al., 1993). Mander's coefficients range from 0 for no colocalization to 1 for complete colocalization and are not influenced by differences in absolute signal intensities in each channel, because pixel intensity in a particular channel is normalized to total pixel intensity across the image for that label (pixels with a value of zero intensity in both channels are considered to be background and are ignored in the computations). The intensity correlation quotient (ICQ) represents the synchrony around which two signals vary and is based on nonparametric sign-test analysis of the product of the differences from the mean (PDM). In an image in which the intensities of staining of two targets vary together, the PDM will be positive. However, if the pixel intensities vary asynchronously, then most of the PDM values will be negative. ICQ values are calculated first by determining the ratio between the number of positive PDM values and the total number of pixel values. From this ratio, 0.5 is subtracted to yield ICQ values distributed between  $-0.5$  and  $+0.5$  where random staining gives an ICQ of  $\sim 0$ , segregated or asynchronous staining gives  $0 > \text{ICQ} \geq -0.5$ , and dependent or synchronous staining yields  $0 < \text{ICQ} \leq +0.5$  (Li et al., 2004).

**Statistical analysis.** Values were expressed as mean  $\pm$  SEM. Statistical significance of differences was assessed with the Student's  $t$  test for unpaired samples from different, independent experiments. Differences were assessed for significance at  $p < 0.01$  based on nonpaired Student's  $t$  test. Statistical assessment used in colocalization image analyses was performed according to the data distribution; normally distributed data were analyzed using two-tailed  $t$  tests, and non-normally distributed data were analyzed using the Mann–Whitney  $U$  test ( $p < 0.05$  was considered significant).

## Results

### Human $\alpha 7$ -nAChRs interact with APC in SH-EP1-h $\alpha 7$ cells

Initial studies to test for possible interactions between  $\alpha 7$ -nAChRs and proteins involved in the *Wnt* signaling system used SH-EP1 cells stably expressing the human nAChR  $\alpha 7$  subunit (SH-EP1-h $\alpha 7$  cells) (Zhao et al., 2003). Western analyses demonstrated the expression of several proteins involved in the *Wnt* pathway. SH-EP1-h $\alpha 7$  cells express Fzd (Frizzled) receptors,

which are the first targets of the *Wnt* canonical ligands, and different DVL isoforms, which are proteins that play an essential role in the dissociation of  $\beta$ -catenin from the destruction complex (Aberle et al., 1997).  $\beta$ -Catenin, the main effector of the *Wnt* pathway, and axin, APC, and GSK-3 $\beta$ , which are different components of the  $\beta$ -catenin destruction complex (Fig. 1Aa), were also found to be expressed in SH-EP1-h $\alpha 7$  cells (data not shown).

To study the possible interaction of  $\alpha 7$ -nAChRs with components of the *Wnt* pathway, we assessed levels of coimmunoprecipitation of  $\alpha 7$ -nAChRs with proteins that play important roles in the regulation of the *Wnt* pathway.  $\alpha 7$ -nAChRs were found to interact with APC but not with  $\beta$ -catenin, DVL-3, or GSK-3 $\beta$  under control conditions (data not shown). To evaluate the ability and specificity of anti-nAChR  $\alpha 7$  subunit antibody to recognize  $\alpha 7$ -nAChR expressed in SH-EP1-h $\alpha 7$  cells and not other proteins, we compared immunoblots performed using extracts from nAChR-null SH-EP1 cells and transfected SH-EP1-h $\alpha 7$  cells and found labeling of a specific protein band at the appropriate molecular mass only in SH-EP1-h $\alpha 7$  cells (data not shown).

These results validated some of the reagents to be used, indicated that human  $\alpha 7$ -nAChRs are able to interact with APC in the *Wnt* signaling pathway in SH-EP1-h $\alpha 7$  cells, and stimulated us to explore these relationships in neurons.

### $\alpha 7$ -nAChRs interact with APC in hippocampal neurons

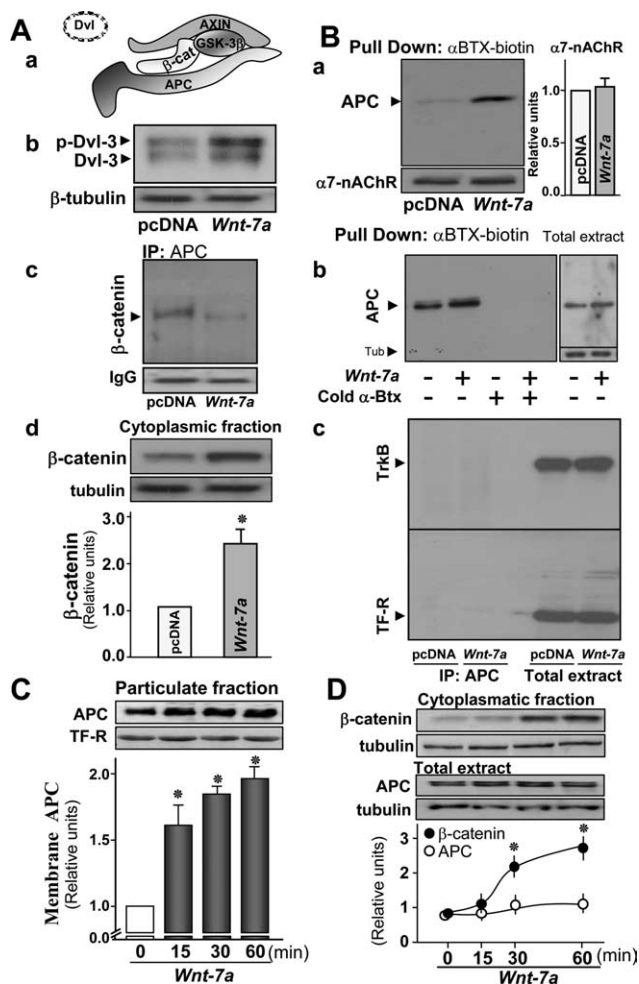
Activation of canonical *Wnt* signaling leads to  $\beta$ -catenin stabilization and translocation into the nucleus to activate target genes (Nusse and Varmus, 1992), but the role of APC outside the destruction complex is not known. In rat hippocampal neurons, APC has been shown to be localized in the cytoplasm, in which it normally forms complexes with several proteins that lead to  $\beta$ -catenin degradation (Aberle et al., 1997; Brakeman et al., 1999). Hippocampal neurons also express other members of the *Wnt* signaling pathway and  $\alpha 7$ -nAChRs (Fabian-Fine et al., 2001; Fariás et al., 2004).

A series of studies was done to confirm the presence of functional *Wnt* signaling in hippocampal neurons and to verify the presence of *Wnt* ligand in conditioned media used to bathe HEK-293 cells transfected to express *Wnt-7a* and its absence in control media used to bathe HEK-293 cells transfected with an empty pcDNA vector. *Wnt* ligands able to activate the *Wnt* signaling pathway induce a phosphorylation-dependent mobility shift of DVL (Cong et al., 2004; Gonzalez-Sancho et al., 2004; Schulte et al., 2005). When hippocampal neurons were incubated with conditioned medium containing *Wnt-7a* ligand for 1 h, a shift was observed from a fast-migrating form evident in control samples ("pcDNA") to a slower-migrating phosphorylated form of DVL-3 (Fig. 1Ab). As a consequence of DVL phosphorylation after *Wnt* pathway activation,  $\beta$ -catenin accumulates (Aberle et al., 1997). In hippocampal neurons, we also observed a twofold increase in  $\beta$ -catenin levels after exposure to *Wnt-7a* (Fig. 1Ad).

To further assess the integrity of the *Wnt* signaling pathway in hippocampal neurons and functional activity of *Wnt-7a* in conditioned medium, we determined the extent of interaction between APC as the immunoprecipitation target and  $\beta$ -catenin as assessed by Western analysis. In hippocampal neurons treated with *Wnt-7a* for 1 h, there was dissociation of  $\beta$ -catenin from APC relative to levels of association under control conditions (Fig. 1Ac).

To evaluate whether endogenous  $\alpha 7$ -nAChRs interact with endogenous APC in rat hippocampal neurons, we performed an





**Figure 1.** APC dissociates from the  $\beta$ -catenin cytoplasmic complex and associates with  $\alpha 7$ -nAChRs and membrane compartments in hippocampal neurons in the presence of *Wnt-7a*. **Aa**, Schematic representation of the  $\beta$ -catenin destruction complex. **Ab**, Western blot analysis after *Wnt-7a* treatment of hippocampal neurons for 1 h reveals increased phosphorylation of DVL-3 normalized for sample loading with tubulin (representative of  $n = 3$ ; pcDNA represents the control condition using conditioned medium without secreted *Wnt-7a* from transfected cells). **Ac**, **Ad**, Immunoprecipitation (IP) of APC and Western detection of  $\beta$ -catenin indicates that the  $\beta$ -catenin/APC complex dissociates after *Wnt-7a* treatment under conditions in which total  $\beta$ -catenin is not altered (**Ac**; representative of  $n = 3$ ), whereas *Wnt-7a* induces  $\beta$ -catenin stabilization in the cytoplasmic fraction marked by the presence of and normalized for sample loading with tubulin (**Ad**;  $n = 4$ ; bar graph shows quantitation). **Ba**, **Bb**, APC interaction as determined by Western analysis with  $\alpha 7$ -nAChRs and assessed after pull down of  $\alpha 7$ -nAChRs using biotinylated  $\alpha$ -Btx/streptavidin-agarose is increased after exposure to *Wnt-7a* relative to pcDNA controls and normalized for total  $\alpha 7$ -nAChRs recovered, which is unchanged by ligand exposure (**Ba**;  $n = 4$ ; bar graph shows  $\alpha 7$ -nAChR quantitation) and is prevented in the presence of excess, fluid-phase Btx under conditions in which total APC levels are not altered (**Bb**;  $n = 3$ ). **Bc**, Immunoprecipitation of APC and detection by Western blot of associated TrkB receptor or TF-R reveals no nonspecific receptor interaction with APC under conditions in which total receptor numbers are high and unchanged by *Wnt-7a* exposure. **C**, Subcellular fractionation and Western blot of APC normalized to transferrin receptor levels indicates increases in APC localization to the particulate fraction in a time-dependent form in the presence of *Wnt-7a* ( $n = 4$ ). **D**, Western blot analysis of  $\beta$ -catenin levels in the cytoplasmic fraction and of APC in total extracts indicates that  $\beta$ -catenin levels increase and become stabilized at 30 min and that total APC levels are not affected over the same time course of *Wnt-7a* treatment ( $n = 4$ ; normalized to  $\beta$ -tubulin levels). Data are the mean  $\pm$  SEM of three to six independent experiments, expressed as fold increase over control cells. In all figures,  $*p < 0.01$  by nonpaired Student's  $t$  test.

immunoprecipitation of the  $\alpha 7$ -nAChR and detection of APC by Western analysis. We observed an interaction between  $\alpha 7$ -nAChRs and APC (data not shown). However, because of concerns that anti-nAChR  $\alpha 7$  subunit antibodies might engage in

nonspecific interactions with other proteins in mouse brain extracts (Herber et al., 2004), we also assessed  $\alpha 7$ -nAChR/APC interactions using biotinylated  $\alpha$ -Btx to pull down intact toxin-binding  $\alpha 7$ -nAChRs from hippocampal neurons and assess their interaction with APC. Under basal conditions (data not shown) or in the presence of control medium (pcDNA) (Fig. 1*Ba*, first lane and first column), we detected APC in  $\alpha 7$ -nAChR complexes, as was indicated in the coimmunoprecipitation assays.

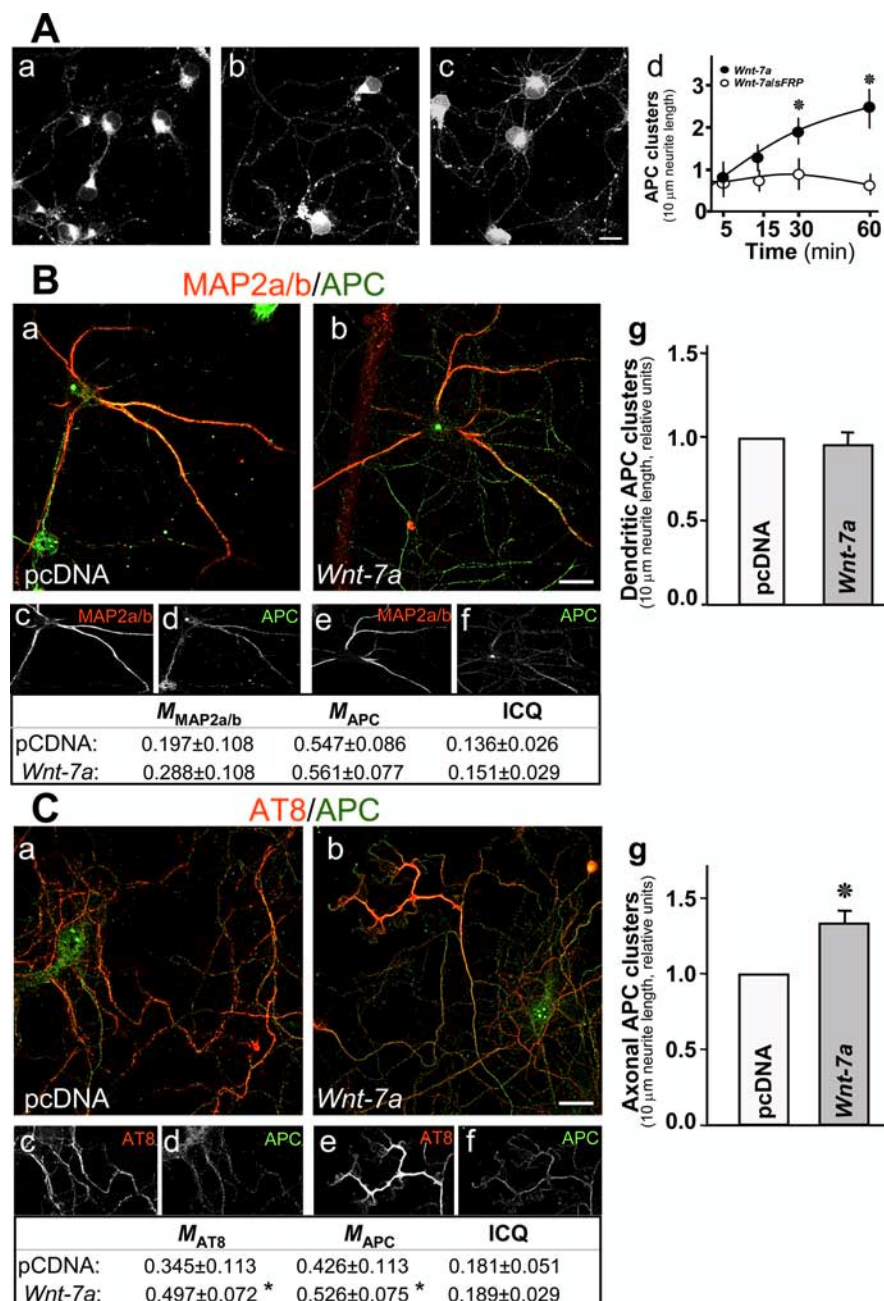
To determine whether activation of *Wnt* signaling promotes stronger interaction between  $\alpha 7$ -nAChRs and APC, hippocampal neurons were incubated for 1 h in the presence of *Wnt-7a* ligand. This treatment specifically (relative to controls) (Fig. 1*Ba*, first lane and first column) induced stronger association of APC with  $\alpha 7$ -nAChRs (Fig. 1*Ba*, second lane and second column) under conditions in which total APC (Fig. 1*Bb*, right, *D*) and total  $\alpha 7$ -nAChR level (Fig. 1*Ba*) remained unchanged. If  $\alpha 7$ -nAChR interaction with biotinylated  $\alpha$ -Btx was inhibited in the presence of excess unlabeled toxin (Fig. 1*Bb*) or nicotine (data not shown), APC was not isolated as assessed by immunoblots in the streptavidin-Sepharose samples that lacked immobilized  $\alpha 7$ -nAChR. To further demonstrate specificity of interactions between  $\alpha 7$ -nAChRs and APC, we immunoprecipitated APC and used Western analysis to assess APC interactions with receptors for transferrin or Trk-B, both of which are present in hippocampal neuron plasma membranes. No interactions between APC and Trk-B or transferrin receptors were detected in either *Wnt-7a*-treated or control neurons, even under conditions in which blots were overexposed with regard to receptor levels in total extracts to enhance the possibility of visualizing these interactions (Fig. 1*Bc*).

These results show that the canonical *Wnt-7a* ligand induces the dissociation of APC from the  $\beta$ -catenin cytoplasmic complex and the association of APC with  $\alpha 7$ -nAChRs in hippocampal neurons.

### APC is associated with membranes and forms clusters in hippocampal neurites.

Having demonstrated that APC interacts with  $\alpha 7$ -nAChRs, the possible association of APC with membranes was studied in rat hippocampal neurons as a function of time of exposure to *Wnt-7a*. Particulate fractions were obtained by subcellular fractionation, and *Wnt-7a* was found to induce the association of APC with membrane-enriched compartments (as assessed by the presence of transferrin receptor) in a time-dependent manner (Fig. 1*C*). Fifteen minutes of incubation with *Wnt-7a* induced a 1.6-fold increase in membrane-bound APC, reaching plateau values of twofold over control after 60 min of *Wnt* ligand exposure (Fig. 1*C*). Under the same conditions, the total levels of APC did not change (Fig. 1*D*). The stabilization of  $\beta$ -catenin in cytoplasmic fractions marked by the presence of tubulin was observed after 30 min of *Wnt-7a* treatment and was maintained at 60 min of *Wnt* ligand exposure (Fig. 1*D*). The observation that APC is dissociated from  $\beta$ -catenin and that the APC association with membranes precedes  $\beta$ -catenin stabilization could indicate that interactions between APC and  $\alpha 7$ -nAChR do not require association of APC with  $\beta$ -catenin.

We next sought to determine whether *Wnt-7a* induces changes in the localization of APC in hippocampal neurons as evaluated by immunofluorescence. In control cells, APC was found mainly in neuronal soma, with a weak localization in neurites (Fig. 2*Aa*). Remarkably, *Wnt-7a* treatment induced the relocalization of APC to neurites, in which APC appeared in the form of clusters (Fig. 2*Ab,Ac*). As a test of specificity of these



**Figure 2.** Wnt-7a induces clustering of APC in neurites. **A**, Immunofluorescence for APC indicates that APC clusters as puncta in neurites and that these APC aggregates increase after Wnt-7a treatment for 0 min (**Aa**), 15 min (**Ab**), and 60 min (**Ac**). Scale bar, 10  $\mu$ m. **Ad**, Quantification of APC puncta per 100  $\mu$ m of neurites demonstrates time dependence for increased APC Wnt-7a-induced puncta and sensitivity of puncta formation to cotreatment with sFRP. Data are the mean  $\pm$  SEM of six independent experiments performed in duplicate. **B**, Double immunofluorescence for APC and MAP2a/b shows that, under control conditions (pCDNA), APC is localized in the soma and dendrites (**Ba**, **Bc**, **Bd**), whereas Wnt-7a treatment induces neuritic localization of APC but not to MAP2a/b-positive dendrites (**Bb**, **Be**, **Bf**). Table, Colocalization analysis ( $M$ ) and ICQ values. **Bg**, The number of dendritic APC clusters is not affected by Wnt-7a treatment relative to control conditions. Scale bar, 10  $\mu$ m. **C**, Double immunofluorescence for APC and p-Tau recognized by AT8 antibody shows that Wnt-7a induces APC relocation to p-Tau-positive axons (**Cb**, **Cc**, **Cd**) relative to control conditions (pCDNA; **Ca**, **Cc**, **Cd**; 3 independent experiments performed in triplicate). Table, colocalization analysis ( $M$ ) and ICQ values. **Cg**, Wnt-7a treatment increases the number of APC clusters in axons relative to control conditions. Scale bar, 10  $\mu$ m.

effects, a strategy was used that involved coinubation of Wnt-7a ligand with sFRP. sFRP recaptures the Wnt ligands, thereby preventing their interaction with cellular membrane-bound Frizzled receptors. Treatment of rat hippocampal neurons with both Wnt-7a and sFRP-conditioned media prevented APC clustering

in neurites (images not shown), indicating the specificity of Wnt ligand action. Quantification of the numbers of APC clusters per 100  $\mu$ m of neurite length showed that Wnt-7a induced a time-dependent appearance of APC clusters on neurites that was prevented in the presence of sFRP (Fig. 2*Ad*).

We used MAP2a/b as a marker to evaluate whether Wnt-7a-mediated translocation and clustering of APC in neurites involves dendrites. Under control conditions, APC is localized in the soma and in some neurites that correspond to MAP2a/b-labeled dendrites (Fig. 2*Ba*, *Bc*, *Bd*). Colocalization analysis indicates that a fraction ( $\sim 20\%$ ) of MAP2a/b colocalized with APC and that a fraction ( $\sim 55\%$ ) of APC colocalized with MAP2a/b (Fig. 2*B*, table). However, there was no significant increase in APC-MAP2a/b colocalization in hippocampal neurons exposed to Wnt-7a (Fig. 2*Bb*, *Be*, *Bf*, table), and there was no significant difference in the synchrony of signal variation (no change in ICQ values, which nevertheless demonstrate overall synchrony in colocalization of staining). Moreover, Wnt-7a treatment for 1 h induced clustering of APC in neurites (Fig. 2*A*), but we did not find an increase in the number of clusters contained in MAP2a/b-positive neurites (Fig. 2*Bg*).

To determine whether the APC clusters induced by Wnt-7a were axonal, we used phosphorylated Tau protein recognized by the AT8 antibody as a marker. Under control conditions, a fraction ( $\sim 43\%$ ) colocalized with AT8-labeled antigen, and a fraction ( $\sim 35\%$ ) of phospho-Tau colocalized with APC (Fig. 2*Ca*, *Cc*, *Cd*, table). Wnt-7a treatment significantly increased the colocalization of APC and phospho-Tau (Fig. 2*Cb*, *Cc*, *Cf*, table). Moreover, Wnt-7a increased APC clusters in AT8-positive neurites relative to control conditions (Fig. 2*Cg*). These results suggest that Wnt-7a induces a functionally relevant relocation of APC to axons in mature hippocampal neurons.

#### Wnt-7a induces APC clustering in presynaptic but not in postsynaptic sites in hippocampal neurons

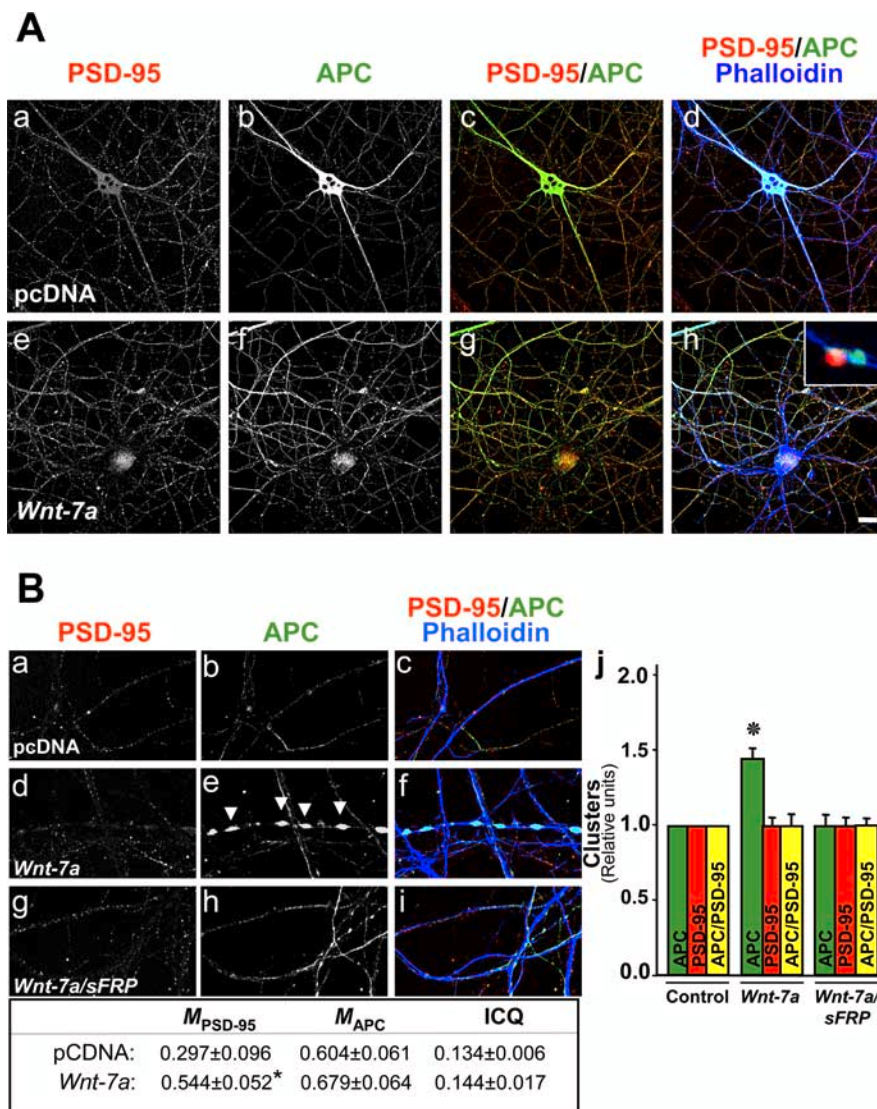
The presence of APC at synapses and more specifically in postsynaptic regions has been demonstrated at the neuromuscular junction and in ciliary ganglion neurons

(Wang et al., 2003; Temburni et al., 2004). To determine whether APC is present in postsynaptic regions of hippocampal neurons, we evaluated whether it is associated with PSD-95, a scaffold protein that, for example, maintains the glutamate receptors anchored to the plasma membrane (Sheng, 2001). Control



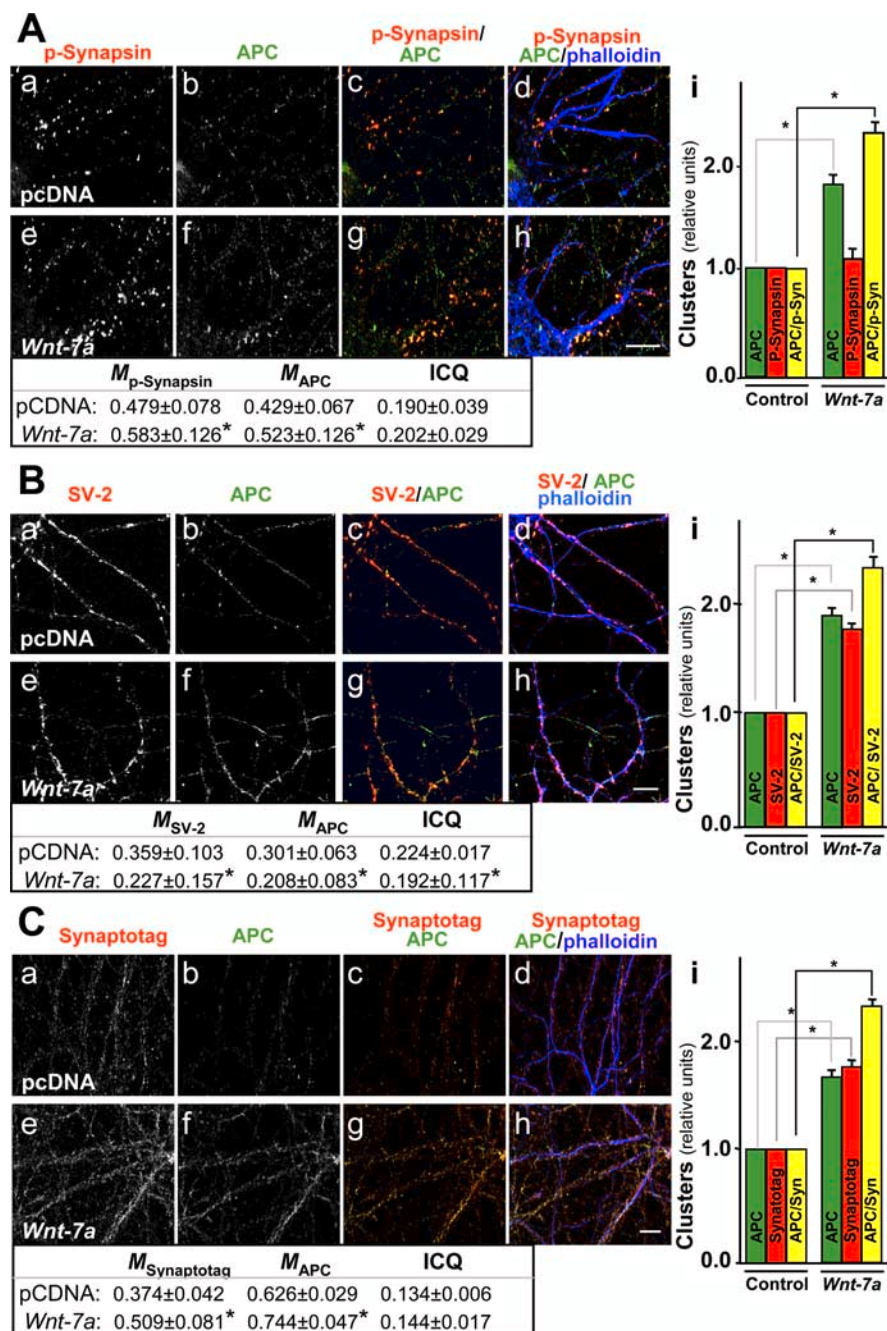
hippocampal neurons exhibit a typical, clustered distribution of PSD-95 in dendrites (Fig. 3*Aa,Ba*). In contrast, APC is only expressed in the soma and in some neurites but not in a clustered pattern (Fig. 3*Ab,Bb*). When hippocampal neurons were exposed to *Wnt-7a* for 1 h, a clear redistribution of APC to clusters in neurites was observed (Fig. 3*Af,Ah,Bc,Bf*). Moreover, we found that the pixel intensity for PSD-95 colocalized with APC increased significantly with *Wnt-7a* treatment but only increased marginally in the colocalization of APC with PSD-95 (Fig. 3*B*, table) (synchrony of signal covariation is indicated by positive ICQ values) despite the fact that the number of APC clusters, but not of PSD-95 clusters or APC/PSD-95 coclusters, increased with *Wnt-7a* treatment (Fig. 3*Bj*). The effect of *Wnt-7a* was specific, because neurite APC cluster formation was prevented by coinubation with sFRP (Fig. 3*Bh,Bi*). Interestingly, treatment with sFRP alone also reduced the detection of APC in neurites (data not shown), suggesting a role for endogenous Wnt ligands in hippocampal neurons. This is consistent with the observation that hippocampus and hippocampal neurons cultures express *Wnt-7a* ligand (W. Cerpa, I. E. Alfaro, G. G. Farias, R. A. Fuentealba, M. J. Metcalfe, J. A. Godoy, C. Bonansco, and N. C. Inestrosa, unpublished observations).

Many of the APC clusters induced by *Wnt-7a* exposure were localized opposite the PSD-95 clusters, suggesting that APC relocates in synapses but not preferentially to postsynaptic excitatory terminals (Fig. 3*Ah*, inset). To determine whether *Wnt-7a* induces presynaptic relocalization of APC, we used p-synapsin-1 and the synaptic vesicle proteins SV-2 and synaptotagmin-1 as markers. Each of these proteins exhibited a typical, clustered pattern in neuritic processes (Fig. 4*Aa,Ad,Ba,Bd,Ca,Cd*). Under control conditions, APC could be observed in some neurites, in which it colocalized and coclustered with p-synapsin, SV-2, and synaptotagmin (Fig. 4*Ab-Ad,Bb-Bd,Cb-Cd*). *Wnt-7a* treatment for 1 h induced higher levels of colocalization of APC with p-synapsin and synaptotagmin in neurites (Fig. 4*Ag,Ah,Cg,Ch*) but decreased the colocalization between APC and SV-2 (Fig. 4*Bg,Bh*). APC and presynaptic marker staining intensities varied in a dependent manner under control or *Wnt-7a* treatment conditions (Fig. 4, tables, positive ICQ values), although the lack of effect of *Wnt-7a* treatment on absolute ICQ values [except the small decrease in APC/SV-2 staining synchrony (Fig. 4, tables)] suggests that the redistribution of APC induced by *Wnt-7a* is not caused by a redistribution of the presynaptic markers. Quantification showed increases of 70–80% in APC clusters in neurons treated with *Wnt-7a*, an



**Figure 3.** APC is not clustered in postsynaptic sites in hippocampal neurons exposed to *Wnt-7a*. **A**, Fluorescence labeling of processes by phalloidin (*Ad, Ah*) and immunofluorescence labeling for APC (*Ab, Af*) or PSD-95 (*Aa, Ae*) are shown in cells subjected to treatments with control (pcDNA; *Aa–Ad*) or *Wnt-7a* (*Ae–Ah*) media. Induction of APC in neurites is observed in the presence of *Wnt-7a* relative to control conditions (*Ab, Ad, Af, Ah*), but its localization at synaptic sites is in apposition to PSD-95-labeled postsynaptic sites, indicating a presynaptic localization (*Ah*, inset). Scale bar, 10  $\mu\text{m}$ . **B**, Three scan zoom images show PSD-95 staining (*Ba, Bd, Bg*), APC staining (*Bb, Be, Bh*), and merged image containing with phalloidin (*Bc, Bf, Bi*) illustrating a modest coclustering of APC with PSD-95 under control conditions (*Ba–Bc, Bj*) but no *Wnt-7a*-induced APC coclustering with PSD-95 (*Bd–Bf, Bj*), indicating that *Wnt-7a*-induced APC clusters are not localized in specialized, postsynaptic sites. *Bh, Bj*, Cotreatment with sFRP prevented *Wnt-7a*-induced clustering of APC. Arrows indicate APC clusters formed in the presence of *Wnt-7a*. Data are the results of four independent experiments performed in duplicate. **B**, Table, Colocalization analysis ( $M$ ) and ICQ indicate an increase in the colocalization of PSD-95 with respect to APC but not vice versa. **Bj**, APC or PSD-95 clusters or coclusters were quantified.

almost 70% increase in SV-2 and synaptotagmin clusters, but no change in p-synapsin clusters (Fig. 4*Ai,Bi,Ci*). Interestingly, *Wnt-7a* exposure increased the number of APC clusters colocalized with p-synapsin, SV-2, and synaptotagmin in hippocampal neurons (Fig. 4*Ai,Bi,Ci*). Because there was a decrease in the absolute colocalization of APC and SV-2 staining intensity but an increase in APC/SV-2 coclustering, we suggest that there is diffuse colocalization of APC and SV-2 under control conditions but that *Wnt-7a* treatment induces an increase in the size of clusters containing these proteins. The results indicate that *Wnt-7a* induces presynaptic relocalization of APC and suggest that *Wnt-7a*, through APC, may play



**Figure 4.** Wnt-7a induces clustering of APC in presynaptic sites in hippocampal neurons. **A**, Immunofluorescence labeling of hippocampal neurons for p-synapsin (**Aa, Ae**) or APC (**Ab, Af**) shows induction in coclustering in the presence of Wnt-7a (**Ag, Ah**) relative to control (pcDNA) treatment (**Ac, Ad**) and as indicated in quantification (**Ai**). **B**, Double labeling of hippocampal neurons for SV-2 (**Ba, Be**) and APC (**Bb, Bf**) shows that Wnt-7a induces coclustering (**Bg, Bh**) relative to control conditions (**Bc, Bd**) and as quantified (**Bi**). **C**, Double labeling for synaptotagmin (Synaptotag; **Ca, Ce**) and APC (**Cb, Cf**) shows induction in coclustering in hippocampal neurons exposed to Wnt-7a (**Cg, Ch**) relative to controls (**Cc, Cd**) and as indicated by quantification (**Ci**). Scale bars, 10  $\mu$ m. Tables, Colocalization analysis ( $M$ ) and ICQ values.

some role in restructuring the presynaptic region in mature hippocampal neurons.

#### Wnt-7a induces presynaptic localization of $\alpha 7$ -nAChRs.

Recent evidence from our laboratory indicates that Wnt-7a induces synaptic vesicle exocytosis from presynaptic regions of hippocampal neurons (Cerpa, Alfaro, Fariás, Fuentealba, Metcalfe, Godoy, Bonansco, and Inestrosa, unpublished observations). Furthermore,  $\alpha 7$ -nAChRs expressed presynaptically on hip-

pocampal neurons modulate transmitter release from GABAergic and glutamatergic terminals (Radcliffe and Dani, 1998; Alkondon and Albuquerque, 2001; Kawai et al., 2002). We evaluated the possible role of Wnt signaling in modulation of the synaptic localization of  $\alpha 7$ -nAChRs and their interaction with presynaptic proteins. Immunoprecipitation of  $\alpha 7$ -nAChRs followed by immunodetection of VAMP-1/2, a component of synaptic vesicles that forms the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) fusion complex enabling vesicle exocytosis (Sytnyk et al., 2004), showed that  $\alpha 7$ -nAChRs interact weakly with VAMP-1/2 in control hippocampal neurons (Fig. 5A). However, treatment for 1 h with Wnt-7a induced a strong association of  $\alpha 7$ -nAChRs with VAMP-1/2 (Fig. 5A). Under similar conditions, total levels of VAMP-1/2 were not changed. Moreover, pull-down assays involving biotinylated  $\alpha$ -Btx-mediated capture of  $\alpha 7$ -nAChR showed increased association of receptor with p-synapsin under conditions in which total levels of p-synapsin remained the same (Fig. 5A).

Evaluation of the interaction between  $\alpha 7$ -nAChRs and SV-2 under control conditions demonstrated colocalization of these proteins (Fig. 5Ba–Bc, Ca–Cc). Wnt-7a exposure induced a decrease in the fraction of  $\alpha 7$ -nAChRs that colocalized with SV-2 (Fig. 5Bd–Bf, Cd–Cf). Quantification showed a decrease in the number of  $\alpha 7$ -nAChRs clusters contained in the clusters of SV-2 in Wnt-7a-treated neurons under conditions in which the number of  $\alpha 7$ -nAChR and SV-2 clusters increased almost 70 and 40%, respectively (Fig. 5Cg). An increase in the cocluster size was apparent after Wnt-7a treatment (Fig. 5Cg). Similar results were observed for interactions between  $\alpha 7$ -nAChR and synaptotagmin (Fig. 6B).

PSD-95 was used as marker to evaluate possible interaction and coclustering with  $\alpha 7$ -nAChRs localized postsynaptically and to determine specificity of Wnt-7a-stimulated relocation of  $\alpha 7$ -nAChRs presynaptically. Immunoprecipitation of  $\alpha 7$ -nAChRs and Western immunodetection of PSD-95 demonstrated interaction

between these two proteins in hippocampal neurons under control conditions, but Wnt-7a exposure decreased interaction between  $\alpha 7$ -nAChRs and PSD-95 (Fig. 5D). Coclustering of  $\alpha 7$ -nAChRs with PSD-95 was observed under control conditions (Fig. 5Ea–Ec). However, Wnt-7a exposure did not affect pixel-level colocalization and instead decreased the ICQ value and the number of  $\alpha 7$ -nAChRs contained in PSD-95 clusters (Fig. 5Ed–Eg, table). These effects in the presence of Wnt-7a occurred under conditions in which  $\alpha 7$ -nAChR clusters increased in number and

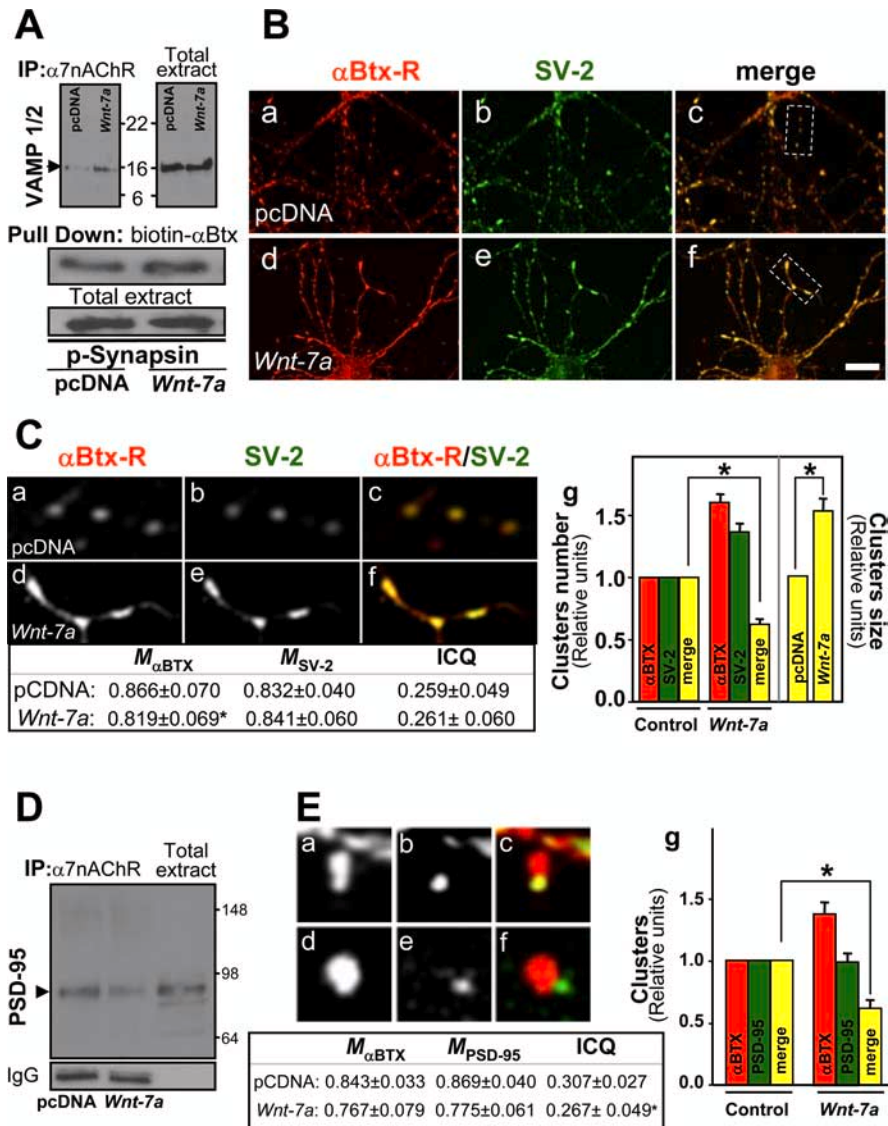


size but in which PSD-95 clusters were not affected (Fig. 5Eg). To assess whether  $\alpha 7$ -nAChRs relocated to presynaptic domains were inserted into the plasma membrane in response to *Wnt-7a* exposure, hippocampal neurons were exposed to *Wnt-7a* ligand for different periods (0–2 h) at 37°C, and the live neurons were then washed free of ligand. Samples were next exposed to biotinylated- $\alpha$ -Btx for 45 min at 4°C to assess cell-surface levels of  $\alpha 7$ -nAChRs while also inhibiting endocytosis of the receptor. *Wnt-7a* exposure induced a time-dependent increase in number of  $\alpha 7$ -nAChRs inserted into the plasma membrane (i.e., isolated by streptavidin-Sepharose pull down of toxin–receptor complexes from lysed cells and visualized on immunoblots), with 1–2 h of treatment producing a 50% increase in surface receptors (Fig. 6A). There was a concomitant decrease in the amount of intracellular  $\alpha 7$ -nAChR (recovered as supernatants from pull-down samples and visualized on immunoblots) (Fig. 6A).

These results indicate that *Wnt-7a* signaling induces a redistribution of  $\alpha 7$ -nAChRs to presynaptic membrane regions.

#### *Wnt-7a* signaling induces presynaptic coclustering of APC and $\alpha 7$ -nAChRs

Because we observed that *Wnt-7a* induced  $\alpha 7$ -nAChR/APC interactions, APC localization to presynaptic sites, and  $\alpha 7$ -nAChR localization to presynaptic sites, we next used triple immunofluorescence to evaluate whether *Wnt-7a* induces presynaptic colocalization of APC and  $\alpha 7$ -nAChRs. Exposure of hippocampal neurons to *Wnt-7a* for 1 h induced the formation of clusters of immunoreactivity for synaptotagmin 1a/b (Fig. 6Ba,Bc,C), a presynaptic protein that acts as a  $\text{Ca}^{2+}$  sensor and interacts with  $\text{Ca}^{2+}$  channels (Sytnyk et al., 2004). An increase in interactions between APC protein (Fig. 6Bb,Bf) and  $\alpha 7$ -nAChRs (Fig. 6Bc,Bg) in synaptotagmin clusters was evident after *Wnt-7a* treatment (Fig. 6Bh, arrows) but not in control samples (Fig. 6Bd, arrows). Quantitative analysis revealed that *Wnt-7a* treatment clearly increased the size of presynaptic synaptotagmin 1a/b clusters by ~85% (Fig. 6Ca). *Wnt-7a* ligand treatment also induced a clear redistribution of APC, as observed previously, and an increase in cluster size to 2.6-fold of control levels (Fig. 6Ca). Moreover, *Wnt-7a* increased the size of  $\alpha 7$ -nAChR clusters to ~2.3-fold of control size (Fig. 6Ca). Quantitative analysis confirmed impressions of a correlation between the sizes of APC and  $\alpha 7$ -nAChR clusters and an increase in the number and size of coclusters in the presence of *Wnt-7a* in the presynaptic region (Fig. 6Cb) (data not shown).



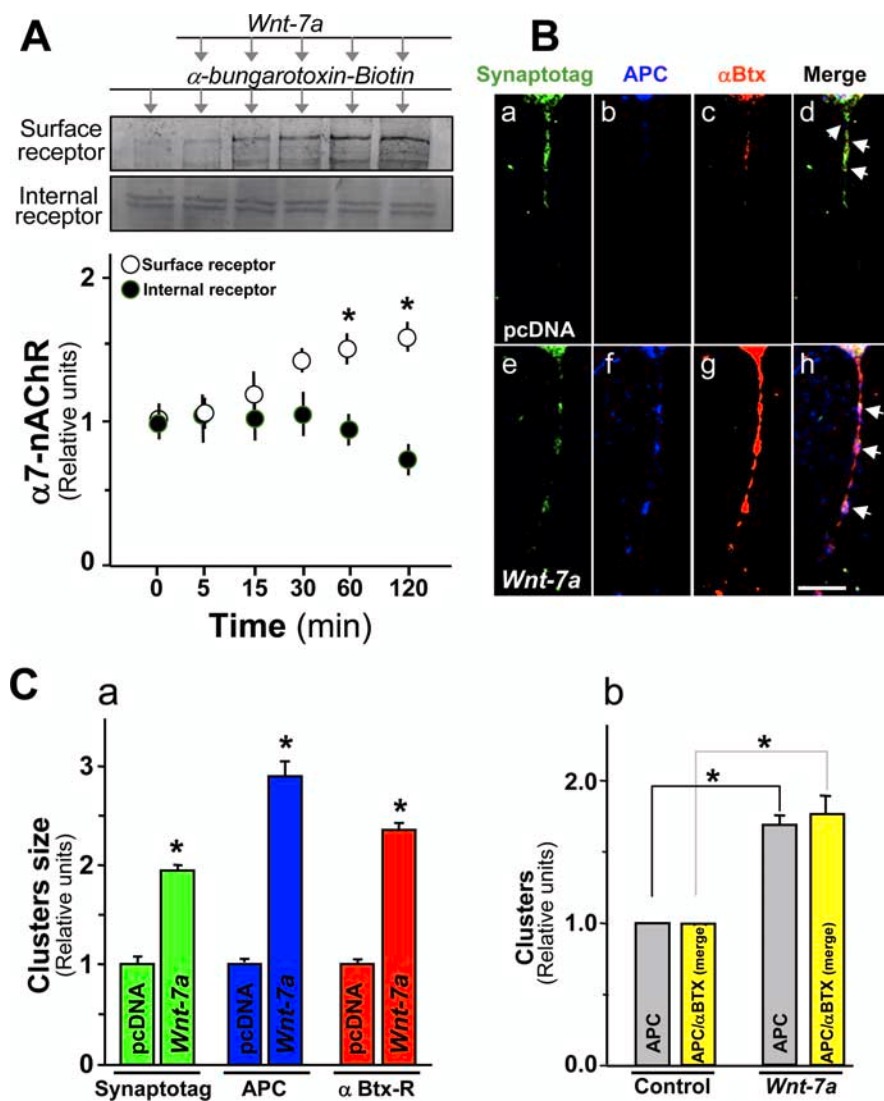
**Figure 5.** *Wnt-7a* increases presynaptic but not postsynaptic relocalization of  $\alpha 7$ -nAChRs in hippocampal neurons. **A**, Immunoprecipitation (IP) of  $\alpha 7$ -nAChRs and Western blot for VAMP-1/2, a synaptic vesicle protein, shows that *Wnt-7a* exposure induces the association of  $\alpha 7$ -nAChRs with VAMP-1/2 under conditions in which the total levels of VAMP-1/2 are not affected ( $n = 3$ ). Pull down of  $\alpha 7$ -nAChRs and Western blot of p-synapsin shows that *Wnt-7a* treatment induces the association of  $\alpha 7$ -nAChRs with p-synapsin, another presynaptic protein ( $n = 3$ ). **B**, Fluorescent rhodamine-labeled  $\alpha$ -Btx ( $\alpha$ -Btx-R; **Ba, Bd**) or SV-2 immunofluorescence (**Bb, Be**) staining in hippocampal neurons treated with control media (pcDNA; **Ba–Bc**) or *Wnt-7a* (**Bd–Bf**) indicates that *Wnt-7a* increases the interaction between  $\alpha 7$ -nAChRs and SV-2 (merged images; **Bc, Bf**). Scale bar, 10  $\mu\text{m}$ . **Ca–Cf**, Magnified views of the boxed regions in **Bc** and **Bf**. **Cg**, *Wnt-7a* induced an increase in the size of  $\alpha 7$ -nAChR and SV-2 coclusters but not the number of clusters. **D**, Immunoprecipitation of  $\alpha 7$ -nAChRs and Western blot for PSD-95 shows  $\alpha 7$ -nAChR association with PSD-95 in control media (pcDNA)-treated samples and that *Wnt-7a* treatment decreases this association (representative of  $n = 3$ ). **E**, Fluorescent rhodamine-labeled  $\alpha$ -Btx (**Ea, Ed**) or PSD-95 immunofluorescence (**Eb, Ee**) staining in hippocampal neurons treated with pcDNA (**Ea–Ec**) or *Wnt-7a* (**Ed–Ef**) indicates that *Wnt-7a* increases the size of  $\alpha 7$ -nAChR clusters but not coclustering with PSD-95. **Eg**, Quantification of  $\alpha$ -Btx staining ( $\alpha 7$ -nAChRs) or PSD-95 clusters and of  $\alpha 7$ -nAChRs contained in PSD-95 clusters, demonstrating a decrease in coclustering after treatment with *Wnt-7a* ligand. **C, E**, Tables, Colocalization analysis ( $M$ ) and ICQ values.

Together, these experiments indicate that the canonical *Wnt-7a* ligand modulates the coclustering of APC and  $\alpha 7$ -nAChR in presynaptic sites and suggest a possible role of APC in the process.

#### Prolonged exposure to *Wnt-7a* increases levels of $\alpha 7$ -nAChRs.

The conventional role of *Wnt* pathway activation involves modulation of gene expression through  $\beta$ -catenin/Tcf-LEF. The ef-





**Figure 6.** APC and  $\alpha 7$ -nAChRs are colocalized in presynaptic regions in hippocampal neurons exposed to *Wnt-7a*. **A**, Effects on surface  $\alpha 7$ -nAChR levels assessed using biotinylated- $\alpha$ -Btx pull down and Western analysis in hippocampal neurons subjected to *Wnt-7a* treatments for different times show increases under conditions in which the fraction of  $\alpha 7$ -nAChRs in the intracellular pool also assessed by immunoblot decreases ( $n = 4$ ). **B**, Rhodamine-labeled  $\alpha$ -Btx fluorescence (**Bc, Bg**) or immunofluorescence for APC (**Bb, Bf**) or synaptotagmin (Synaptotag; **Ba, Be**) in hippocampal neurons treated with control medium (pcDNA; **Ba–Bd**) or *Wnt-7a* (**Be–Bh**) for 1 h indicates that *Wnt-7a* induces clustering of synaptotagmin (**Be**), APC (**Bf**), and  $\alpha 7$ -nAChR (**Bg**). **Bh**, Arrows, *Wnt-7a* elevates colustering of APC and  $\alpha 7$ -nAChR in presynaptic regions. Scale bar, 5  $\mu$ m. **C**, Quantification of size for synaptotagmin (green bars), APC (blue bars), or  $\alpha 7$ -nAChR (identified by  $\alpha$ -Btx-R staining; red bars) clusters contained in neurites of hippocampal neurons (**Ca**) and quantification of cluster numbers for APC and for APC contained in  $\alpha$ -Btx clusters (**Cb**). Data are the mean  $\pm$  SEM of five independent experiments. One hundred clusters per treatment per each independent experiment were evaluated using LSM 5 Image Browser.

effects of shorter-term exposure to *Wnt-7a* on interactions between APC and  $\alpha 7$ -nAChR and relocation to the presynaptic terminal were not accompanied by effects on total levels of  $\alpha 7$ -nAChRs. However, prolonged activation of *Wnt* signaling after 12–24 h of treatment with *Wnt-7a* increased  $\alpha 7$  subunit protein levels in an effect that began to reverse after 48 h of ligand exposure (data not shown). After 24 h of *Wnt-7a* treatments, there was an increase to  $\sim 1.7$ -fold of control levels of nAChR  $\alpha 7$  protein (Fig. 7Aa,Ab). An induction of  $\beta$ -catenin levels indicated that the *Wnt* pathway remained activated at 24 h of *Wnt-7a* exposure (Fig. 7Aa). Immunofluorescence assessments showed that hippocampal neurons treated with *Wnt-7a* for 24 h had elevated expression of  $\alpha 7$ -nAChRs, mainly in neurites (Fig. 7Ba,Bb). Quantitative

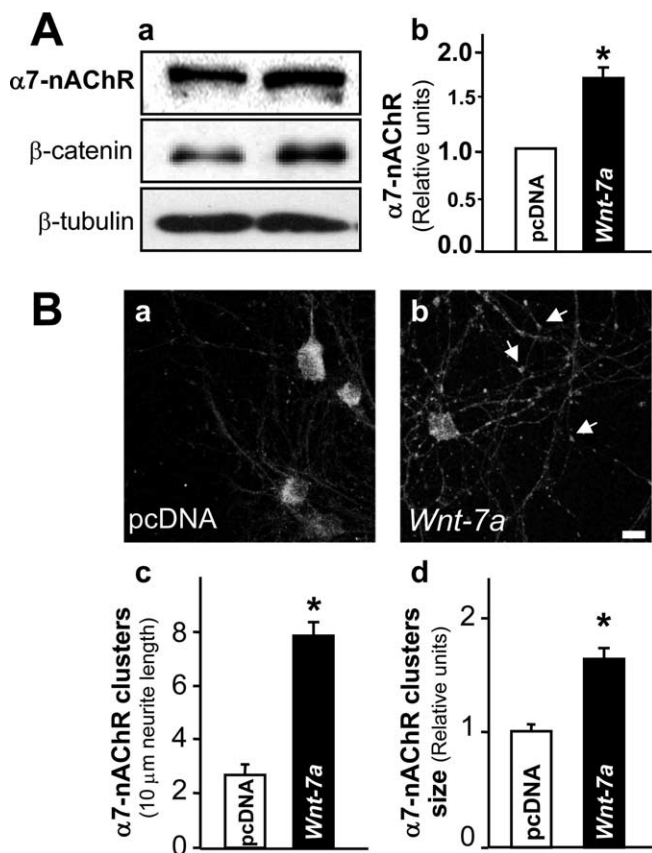
analysis indicated that the increase in the number of  $\alpha 7$ -nAChRs clusters was almost threefold (Fig. 7Bc) and that there was an  $\sim 80\%$  increase in the size of each cluster (Fig. 7Bd) under conditions in which the fluorescence intensity in the soma was not significantly altered by *Wnt-7a* treatment (data not shown). These results suggest that the nAChR  $\alpha 7$  subunit is also a conventional target of canonical *Wnt* action.

#### APC mediates *Wnt-7a*-induced increases in $\alpha 7$ -nAChR presynaptic clustering but not on nAChR $\alpha 7$ subunit expression

To investigate roles of APC in short- and longer-term effects of *Wnt-7a* on clustering of APC and  $\alpha 7$ -nAChR in presynaptic terminals, we examined effects of siRNA knockdown of APC. Mouse hippocampal neurons were cotransfected at 10 d *in vitro* with APC siRNA or control (Ct) siRNA along with GFP protein to allow visualization of transfected neurons and their neurites. Two days later, neurons were exposed to *Wnt-7a* for 24 h, and rhodamine- $\alpha$ -Btx or anti-APC antibodies were used to label  $\alpha 7$ -nAChRs or APC, respectively. Transfection with the APC siRNA construct markedly suppressed APC levels in mouse hippocampal neurons (Fig. 8Ac,Ag,Ak,Ao). Neurons under control conditions (i.e., transfected in unconditioned medium with control siRNA; GFP/Ct-siRNA) showed mainly a somatic localization of  $\alpha 7$ -nAChR and more weakly so in neurites (Fig. 8Aa,Ab). After treatment with *Wnt-7a* for 24 h, neurons transfected with control siRNA exhibited an increase in the neuritic localization of APC and  $\alpha 7$ -nAChR relative to cells treated with unconditioned medium controls (Fig. 8Ae–Ah). Neurons transfected with APC siRNA under control conditions showed an increase in  $\alpha 7$ -nAChR levels relative to neurons transfected with control siRNA and also maintained in unconditioned medium (Fig. 8Ab,Aj), but this increase occurred in soma and not in neurites (Fig.

8Aq). The increase in  $\alpha 7$ -nAChRs is consistent with the nAChR  $\alpha 7$  subunit gene being a conventional *Wnt* target, knockdown of APC (implicated in the degradation of  $\beta$ -catenin) leading to  $\beta$ -catenin accumulation in the cytosol and its translocation to the nucleus, in which it induces *Wnt* target genes.

When hippocampal neurons were transfected with APC siRNA and treated with *Wnt-7a* ligand,  $\alpha 7$ -nAChR levels increased relative to levels in cells transfected with the same construct but maintained in unconditioned medium (Fig. 8Aj,An), but again  $\alpha 7$ -nAChRs were more highly expressed in soma (Fig. 8Am,An,Aq). Complementary studies focusing on neuritic localization of APC and  $\alpha 7$ -nAChRs indicated that neurons transfected with APC siRNA do not have significant levels of neuritic



**Figure 7.** *Wnt-7a* increases nAChR  $\alpha 7$  subunit levels in hippocampal neurons. **A**, Western blot analysis of hippocampal neurons treated with *Wnt-7a* (right) or control (pcDNA; left) medium for 24 h was performed to assess effects on nAChR  $\alpha 7$  subunit,  $\beta$ -catenin, or  $\beta$ -tubulin (normalization control) levels (**Aa**) and showed an increase in  $\alpha 7$  subunit levels (**Ab**;  $n = 5$ ). **B**, Hippocampal neurons exposed for 24 h either to control (pcDNA; **Ba**) or *Wnt-7a* (**Bb**) treatments were analyzed using immunofluorescence to determine the distribution of total expressed  $\alpha 7$ -nAChRs. *Wnt-7a* treatment for 24 h induced preferential localization of  $\alpha 7$ -nAChR clusters in neurites (arrows). Scale bar, 10  $\mu$ m. **Bc**, Quantification of  $\alpha 7$ -nAChR clusters per 10  $\mu$ m of neurite length shows that *Wnt-7a* induces cluster formation in neurites. Data are the mean  $\pm$  SEM of four independent experiments. **Bd**, Quantification shows that *Wnt-7a* induces an increase in the size of clusters containing  $\alpha 7$ -nAChR. Data are the mean  $\pm$  SEM of four independent experiments. One hundred clusters per treatment per each independent experiment were evaluated using LSM 5 Image Browser.

$\alpha 7$ -nAChR or translocation of those sites to neurites after *Wnt-7a* exposure, whereas receptor levels and their localization to neurites are higher under control conditions and increase with *Wnt-7a* exposure in neurons transfected with control siRNA (Fig. 8B). These results confirm the idea that  $\alpha 7$ -nAChR is a *Wnt* target, because APC knockdown is equivalent to persistent *Wnt* signaling as assessed by nAChR  $\alpha 7$  subunit gene overexpression. Furthermore, these results suggest that APC plays a critical role in localizing  $\alpha 7$ -nAChR to presynaptic regions.

## Discussion

nAChR  $\beta$  subunit-mediated interactions have been demonstrated between APC and the muscle-type receptor (Wang et al., 2003) and interactions between APC and nAChRs containing  $\alpha 3$  subunits in ciliary ganglion neurons (Temburni et al., 2004). Here, we show that *Wnt-7a* induces specific interaction between  $\alpha 7$ -nAChRs and APC in rat hippocampal neurons. To our knowledge, this is the first evidence for the modulation of nAChR-APC interactions by canonical *Wnt* signaling.

$\alpha 7$ -nAChRs have been identified at postsynaptic sites, at

which they mediate classic, excitatory neurotransmission, perisynaptic sites, at which they exert a variety of modulatory effects, and presynaptic sites, at which they can modulate neurotransmitter release (Berg and Conroy, 2002). There is a wealth of information on  $\alpha 7$ -nAChRs in the presynaptic modulation of neurotransmitter release (Radcliffe and Dani, 1998; Alkondon and Albuquerque, 2001; Kawai et al., 2002). However, factors controlling the presynaptic localization of  $\alpha 7$ -nAChRs are not well understood.

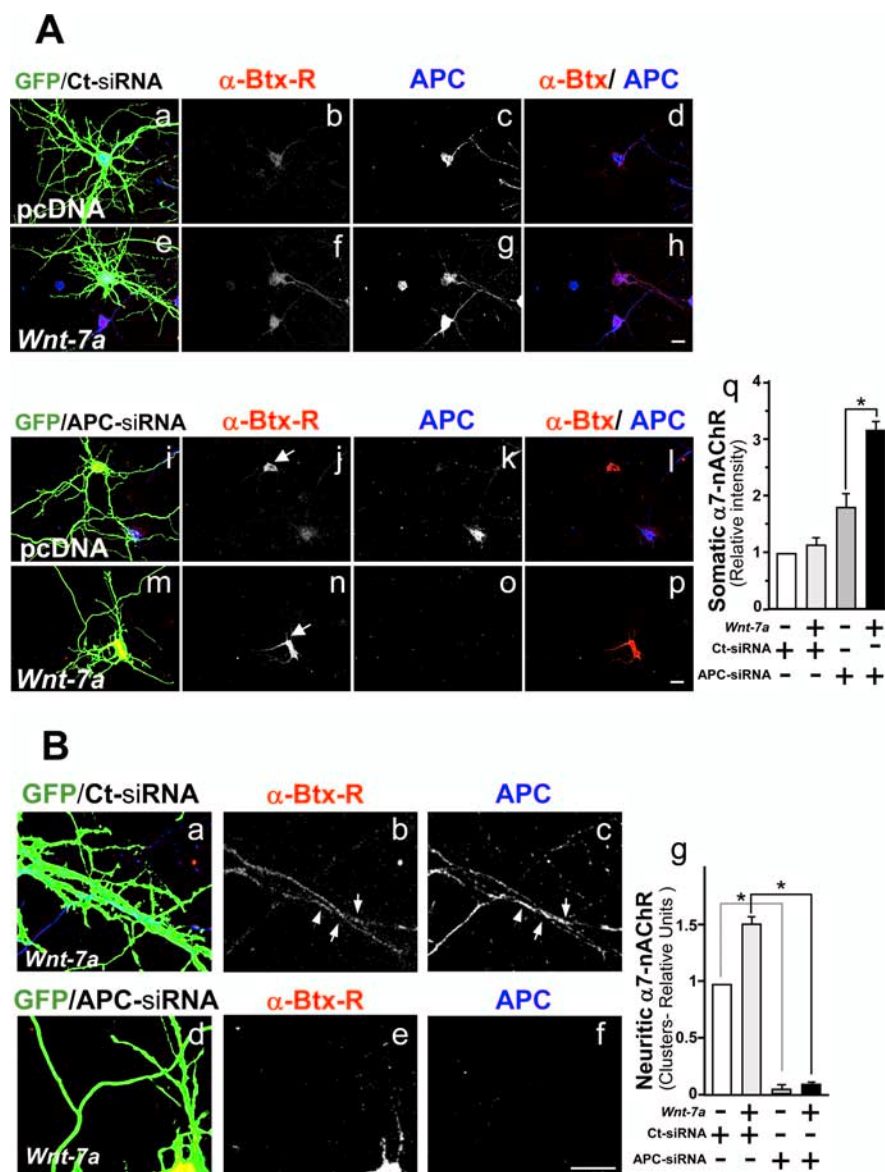
The conventional effects of canonical *Wnt* signaling activation on different *Wnt* target genes are well known. *Wnt* signaling leads to stabilization and cytoplasmic accumulation of  $\beta$ -catenin, which is translocated to the nucleus, in which it activates the transcription of several *Wnt* target genes. There is no study to date, however, that reports on the early and late effects of *Wnt* signaling on target gene transcription in a specific system. The effects of *Wnt* signaling inhibition, however, have been studied using a variety of strategies such as in the presence of an excess of the Frizzled ligand binding domain, Dickkopf, through overexpression of axin, and via the activation of glycogen synthase kinase (van der Heyden et al., 1998; Lustig et al., 2002).

Here, we show that the canonical *Wnt* signaling affects  $\alpha 7$ -nAChRs in rodent hippocampal neurons in two ways: induction of  $\alpha 7$ -nAChR expression and relocalization of  $\alpha 7$ -nAChR to presynaptic sites. APC plays a role in  $\alpha 7$ -nAChR relocalization, because the process is disrupted in the presence of APC siRNA. This effect occurs despite the fact that knockdown of APC, which mimics activation of *Wnt* signaling by freeing  $\beta$ -catenin and thus allowing transcriptional consequences of *Wnt* pathway activation, produces an increase in nAChR  $\alpha 7$  subunits and, hence, total  $\alpha 7$ -nAChR levels. Thus, the nAChR  $\alpha 7$  subunit gene could be another *Wnt* target. Interestingly, *in silico* analysis of the nAChR  $\alpha 7$  subunit gene promoter reveals transcription binding sites for the Tcf-LEF/ $\beta$ -catenin complex, although empirical evidence is needed to demonstrate the functionality of the site.

Recent studies have elucidated nonconventional roles of APC in the *Wnt* signaling pathway beyond its well understood role in regulating breakup of the  $\beta$ -catenin destruction complex. APC can associate with the cytoskeleton through its direct or indirect binding to microtubules (Dikovskaya et al., 2001). In neurons, APC has been shown to regulate the assembly of microtubules critical to axonal growth (Zhou et al., 2004) in a process that is inhibited by  $\beta$ -catenin (Votin et al., 2005). There is also indirect evidence of APC interactions with the actin cytoskeleton (Dikovskaya et al., 2001). Activation of *Wnt* signaling in carcinoma cells also induces the dissociation of APC from the  $\beta$ -catenin destruction complex and APC association with microtubules (Penman et al., 2005). Here, we found that APC dissociated from the  $\beta$ -catenin complex followed by its fast (minutes) association with membrane compartments and relocalization to MAP2a/b-negative, p-Tau-positive neurites corresponding to axons in *Wnt-7a*-treated hippocampal neurons. The magnitude of the increase in the number of APC clusters in neurites assessed using APC staining was higher than the magnitude calculated on the basis of axonal staining using colocalization with the anti-Tau antibody. However, because the AT-8 antibody does not stain all axons uniformly, it is possible that there are some clusters of APC in some sections of axons that are AT-8 negative. The functional consequences of APC in axons are clear in neuronal morphogenesis but still need to be defined in mature neurons.

In ciliary ganglion neurons, APC has been implicated in localization of  $\alpha 3$ -nAChRs to postsynaptic membranes (Temburni et al., 2004). However, in hippocampal neurons, APC colocalizes





**Figure 8.** *Wnt-7a* treatment increases nAChR  $\alpha 7$  subunit levels in an APC-independent manner and alters the neuritic localization of  $\alpha 7$ -nAChRs in an APC-dependent manner. **A**, Mouse hippocampal neurons cotransfected at 10 d *in vitro* with green fluorescent protein and control siRNA (GFP/Ct-siRNA; **Aa–Ah**) or with GFP and anti-APC siRNA (GFP/APC-siRNA; **Ai–Ap**) were treated in the presence of control media (pcDNA; **Aa–Ad**, **Ai–Al**) or *Wnt-7a* (**Ae–Ah**, **Am–Ap**) for 24 h and were stained with rhodamine-labeled  $\alpha$ -Btx ( $\alpha$ -Btx-R; **Ab**, **Af**, **Aj**, **An**) or anti-APC (**Ac**, **Ag**, **Ak**, **Ap**). Arrows show regions of  $\alpha$ -Btx-R stain. GFP images were overexposed to visualize neurites. Scale bar, 10  $\mu$ m. **Aq**, Somatic levels of  $\alpha 7$ -nAChR staining. **B**, Zoom images of *Wnt-7a*-treated (24 h) GFP/Ct-siRNA (**Ba–Bc**) or GFP/APC-siRNA (**Bd–Bf**) mouse hippocampal neurons stained with  $\alpha$ -Btx-R (**Bb**, **Be**) or anti-APC (**Bc**, **Bf**) as well as a quantitation of neuritic  $\alpha 7$ -nAChR clusters (**Bg**). Arrows show clusters of  $\alpha 7$ -nAChR and APC in control transfected neurons. **Aq**, Neurons transfected with APC siRNA show an increase in the soma of  $\alpha$ -Btx-R stain in the presence of control media relative to levels in neurons transfected with control siRNA, and *Wnt-7a* treatment induces an additional increase in somal  $\alpha$ -Btx-R staining in neurons transfected with APC siRNA but not in neurons transfected with control siRNA. **Bg**, In neurons transfected with inactive siRNA and exposed to *Wnt-7a*, an increase in the neuritic localization of  $\alpha 7$ -nAChR is observed relative to similarly transfected neurons in control media, but neuritic  $\alpha 7$ -nAChRs are very low and insensitive to *Wnt* treatment in APC-deficient cells. Data are the mean  $\pm$  SEM of three independent experiments performed in triplicate, expressed as fold increase over control cells.

with the presynaptic marker synaptotagmin (Matsumine et al., 1996). We did find an association between APC and the postsynaptic density protein, PSD-95, in hippocampal neurons, but *Wnt-7a* signaling did not increase coclustering of APC with PSD-95, and closer observation revealed that there was apposition of APC and PSD-95 in synaptic regions but not colocalization at postsynaptic sites. It is possible that *Wnt* signaling through other

proteins regulates some components of the postsynaptic complex, perhaps differentially at excitatory or inhibitory synapses. However, *Wnt-7a*-mediated signaling-induced coclustering of APC with p-synapsin, SV-2, and synaptotagmin strongly implicates APC in regulation of presynaptic complexes in hippocampal neurons, thereby expanding the realm of possibilities regarding roles of APC in neuronal function.

*Wnt* signaling has been implicated in hippocampal neurogenesis (Lie et al., 2005) and in neurotransmitter release, the modulation of synaptic activity, and synaptogenesis in mossy fibers (Ahmad-Annur et al., 2006). Moreover, adult rat hippocampus and mature hippocampal neurons in culture express *Wnt* ligand, and we have observed that *Wnt-7a* induces hippocampal neuronal synaptic vesicle exocytosis (Cerpa, Alfaro, Fariás, Fuentelba, Metcalfe, Godoy, Bonansco, and Inestrosa, unpublished observations). Here, we demonstrate that *Wnt-7a* signaling induces the clustering of APC and  $\alpha 7$ -nAChR on mature neurons in presynaptic membranes as indicated by their counteraction with SV-2, p-synapsin, VAMP-1/2, and synaptotagmin 1a/b. This suggests that *Wnt-7a* may dynamically modulate neurotransmitter release by altering  $\alpha 7$ -nAChRs levels at synaptic terminals, thereby contributing to synaptic plasticity. *Wnt-7a* exposure also affects the number and cluster size of several other essential, presynaptic proteins involved in neurotransmitter release, providing ways to affect synaptic function and plasticity in addition to or independent of effects on  $\alpha 7$ -nAChRs. APC plays an essential role in localization of  $\alpha 7$ -nAChR at the synapse, but there are some APC clusters that do not contain  $\alpha 7$ -nAChRs, so APC may also facilitate localization of other proteins to synapses at sites distinct from those at which  $\alpha 7$ -nAChRs cluster. Nevertheless, whether in maturing or mature neurons,  $\alpha 7$ -nAChRs could be an important synaptic target of *Wnt* signaling and/or an effector of the *Wnt* pathway.

Interestingly, whereas clusters of SV-2 or synaptotagmin containing  $\alpha 7$ -nAChR increased in size after *Wnt-7a* exposure, the number of  $\alpha 7$ -nAChR/SV-2 coclusters decreased, suggesting that subtle processes

are involved in  $\alpha 7$ -nAChR assembly, localization, and maintenance of position. *Wnt* ligands are a family of secreted glycoproteins encoded by a family of  $\sim 20$  conserved genes in humans and other mammals (Moon et al., 2002), and we propose that different isoforms could be responsible for finer control of synaptic localization of  $\alpha 7$ -nAChRs.

AD is a neurodegenerative pathology characterized by an ex-

tracellular accumulation of A $\beta$  in the form of fibril aggregates that form senile plaques (Coyle et al., 1983; Selkoe, 2001; Palmer, 2002). The cholinergic impairment observed in AD patients is associated with a decrease in  $\alpha 7$ -nAChR levels (Banerjee et al., 2000) and a loss of synaptic contacts (Lacor et al., 2004). Therapies focused on the induction of cholinergic function for neuroprotection of A $\beta$  have not been effective in AD, possibly because of the decrease in  $\alpha 7$ -nAChRs in AD patients.

Neuronal death induced by A $\beta$  *in vitro* can be prevented by exposure to canonical Wnt ligand (Alvarez et al., 2004), defects in Wnt signaling have been implicated in AD (De Ferrari and Inestrosa, 2000; Garrido et al., 2002; Caricasole et al., 2003; Fuentealba et al., 2004), and a loss of signaling through the  $\beta$ -catenin-TCF pathway increases neuronal vulnerability to A $\beta$ -induced apoptosis (Zhang et al., 1998). Recent evidence suggests that A $\beta$  induces a deregulation of Wnt signaling components (De Ferrari et al., 2003; Alvarez et al., 2004; Fariás et al., 2004; Quintanilla et al., 2005). However, the mechanisms by which Wnt signaling plays a role in AD pathology remain unclear. This is relevant in the context of the present work, because we found that  $\alpha 7$ -nAChR is a target of the Wnt pathway. Therapies aimed at activating Wnt signaling may possibly be effective in the treatment of AD, especially if they prevent loss of  $\alpha 7$ -nAChRs and other important synaptic proteins.

In conclusion, the connection between  $\alpha 7$ -nAChRs and the Wnt pathway reported here provides new insights into the mechanisms relevant to maintenance of synaptic activity in health and disease.

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